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APPLICATION NUMBER: *60/372,432*
FILING DATE: *April 16, 2002*

PRIORITY DOCUMENT
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Box Provisional Application
PTO/SB/16(8-00)

J1131 U.S. PTO
04/16/02

J1011 U.S. PTO
60/372432
04/16/02

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. § 1.53 (c).

Filing Date	April 16, 2002	Docket No.	2786-0210P

INVENTOR(s)/APPLICANT(s)

Given Name (first and middle [if any])	Last Name	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
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☐ Additional inventors are being named on the separately numbered sheets attached hereto

TITLE OF THE INVENTION (280 characters max)

METHOD, SYSTEM AND KIT FOR DETECTING AN ANALYTE IN A SAMPLE

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages:* 15
☒ Drawing(s) *Number of Sheets:* 2
☐ Application Data Sheet. See 37 CFR 1.76.
☐ Other (specify):______

METHOD OF PAYMENT *(check one)*	PROVISIONAL FILING FEE
☐ Applicant claims small entity status. See 37 CFR 1.27.	☐ Small Entity ($80.00)
☒ A check or money order is enclosed to cover the Provisional filing fees.	☒ Large Entity ($160.00)
☐ The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number 02-2448, if necessary.	

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

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MSW/sh
2786-0210P

(Rev. 02/20/02)

METHOD, SYSTEM AND KIT FOR DETECTING AN ANALYTE IN A SAMPLE

FIELD OF THE INVENTION

The present invention concerns diagnostics by the use of light microscopy and image analysis.

BACKGROUND OF THE INVENTION

Early detection of an active disease in a patient is an essential factor for a successful treatment as it enables to slow down the progress and at times cure patients from a disease provided that a rapid and correct diagnosis is established.

Traditional methods for the detection of infectious diseases include serology assays, e.g. complement fixation (CF), indirect and direct fluorescent antibody (IFA and DFA, respectively), enzyme-linked immunosorbent assay (ELISA) and latex agglutination; culturing assays in which the infectious microorganism recovered from a patient during acute infection is cultured and then identified; and assays involving the use of monoclonal antibodies specific against the infectious agent.

Flow cytometry analysis is also used for disease detection and involves the measuring of certain physical and chemical characteristics of cells or particles, including cell size, shape and internal complexity or any other cell component that can be detected by a fluorescent compound, as the cells or particles travel in suspension one by one past a sensing point. The use of flow cytometry for detection methods has been described, for example in WO99/47933. This publication describes a method for the detection of surface antigens by contacting an antibody-

coupled bead with a test sample and, if the target antigen is present in the sample, a bead-antibody-antigen complex is thus formed and detected by flow cytometry.

US 6,159,748 describes a kit for the detection of antibodies in serum samples using a flow cytometer. In particular, the kit is provided with beads coated with a series of antigens, each having a different bead size and carrying a different antigens. The beads are used for the detection of different antibodies, including auto-antibodies.

As appreciated by those versed in the art, when utilizing a flow cytometry instrument, the cell sample preparation, data collection and data analysis must be performed by a specially trained technician. The flow cytometry instrument includes a laser and complex optical system, a high-power computer and electrical and fluidic systems. The component systems of the flow cytometry instrument must be properly maintained and calibrated on a regular and frequent basis. The high cost of the instrument and the expertise required to correctly operate such instrument render detection by flow cytometry convoluted and expensive. Evidently, this rational also applies to many other tests and instruments, including, *inter alia*, Enzyme-Linked Immunosorbent Assay (ELISA).

Thus, there is a need for the providence of a rapid, sensitive and easy-to-perform method of detecting *in vitro* analytes in a sample obtained from a subject as well as for the monitoring of a disease.

DESCRIPTION OF THE INVENTION

The present invention provides a rapid and easy method of diagnosing in vitro an analyte in a sample obtained from a subject, without the need of any sophisticated equipment. Moreover, the method of the invention is sensitive and allows detection at an early stage of an oncoming disease and provides a tool to follow a patient from onset to the end of the specific disease and to monitor the effectiveness of a chosen treatment against the disease.

Thus, according to one aspect of the invention there is provided a method for detecting an analyte in a sample, the analyte comprising at least two recognition sites, the method comprising:

(a) providing a capturing agent comprising at least two capturing moieties capable of binding to a recognition site of said analyte;

(b) contacting the sample with said capturing agent under conditions so as to allow the analyte, if present in the sample, to associate with the agent via binding of a recognition site of said analyte to a capturing moiety of said agent, thereby forming agent-analyte particulate;

(c) obtaining an optical image of the sample by a light microscope (alternatively lens of a light microscope may be used, without the rest of it's components); and

(d) determining from the image the absence or presence of particulates formed as a result of the binding between said analyte and said capturing agent, the presence of particulates in the sample indicating the presence of said analyte in the sample.

The term *"detect"* or *"detection"* as used herein refers collectively to both a qualitative and quantitative determination of the presence of an analyte in a sample. Thus, the method of the present invention also provides analytical detection of a target analyte in a sample. According to this embodiment, i.e. the quantitative detection, there is provided a method for the quantitative detection of an analyte in a sample, the analyte comprising at least two recognition sites, the method comprising:

(a) providing a capturing agent comprising at least two capturing moieties capable of binding to a recognition site of said analyte;

(b) contacting the sample with said capturing agent under conditions so as to allow the analyte, if present in the sample, to associate with the capturing agent via binding of a recognition site of said analyte to a capturing moiety of said agent, thereby forming agent-analyte particulates;

(c) obtaining an optical image of the sample by a light microscope; and

(d) determining from the image size distribution of the particulates formed as a result of the binding between said analyte and said capturing agent, the size distribution of the particulates indicating the level of analyte in the sample.

According to the invention, the binding of an analyte to the capturing agent results in the formation of a recognition complex. The capturing agent and the analyte, being a recognition partner, constitute together a recognition couple. The recognition couple may, for example, be one of the couples selected from the group of receptor-ligand, sugar-lectin, antibody-antigen (the term *"antibody"* should be understood as referring to a polyclonal or a monoclonal antibody, to a fraction of an antibody comprising the variable, antigen-biotin binding portion, etc.).

The "***analyte***" according to a first aspect of the invention refers to any cellular or microorganism component such as proteins (e.g. antibodies, cytokines, receptors), glycoproteins, peptides, low molecular weight compounds, the detection of which in a sample obtained from a subject is indicative of whether the subject has a specific disease or disorder. The analyte according to this aspect of the invention contains at least two binding sites (recognition sites) to which two individual capturing agents may bind. The results of binding to each binding site of the analyte to an individual capturing agent thus results in the formation of aggregates (referred to herein also by the term "*particulates*") detectable by light microscope.

According to a second aspect, the analyte refers to particles presenting on their surface at least two binding (recognition) sites. For example, the analyte may include antigen-presenting particles, e.g. antigen presenting cells, viruses or other infectious agents, which present on their surface specific antigens to which the capturing agent binds.

The term "***capturing agent***" according to the invention refers to any bi or multifunctional agent, which may bind, respectively, to two or more analytes in a sample. Accordingly, the agent includes dimmeric, trimeric or multimeric molecules presenting respectively two, three or more capturing sites which can bind independently to an analyte. For example, the agent may be a dipeptide or diprotein bridged by a linker. According to a preferred embodiment the capturing agents are microbeads coated with specific capturing moieties.

According to one embodiment of the invention, the capturing agent is an antigen comprising at least two antigenic epitopes and the analyte is an antibody comprising at least two binding sites to which antigenic epitopes of different capturing agents binds, or *vice versa,* the agent is an antibody comprising at least two recognition sites and the analyte is an antigen comprising at least two antigenic epitopes or a particle presenting on its surface at least two antigenic epitopes to which two different antibodies bind.

According to the second aspect of the method of the invention, the capturing agents are microbeads coated with capturing moieties. The microbeads may comprise on their sensing interface a single type of capturing agent or several types of capturing agents so as to enable the use of the coated microbeads in different detection assays. Microbeads which used according to the invention may be made of polymer such as polystyrene, latex etc. which are coated with the capturing agent either by simple adorption, by the aid of cross-linking agents or any other method of conjugating the capturing agent to the microbeads, as known by those versed in the art.

The ***sample*** according to the invention refers to any body fluid, including blood (plasma and serum), saliva, urine or cellular moieties derived from body fluids (e.g. blood cells), or cellular components which may be obtained from a tissue or from body cavities and then suspended in a suitable medium for detection by the method of the present invention.

The invention also provides a system for detecting an analyte in a sample, the system comprising:

(a) capturing agents comprising two or more capturing moieties for binding to said analyte;

(b) a light microscope coupled to an optical image acquisition device;

(c) an image analysis device.

According to an alternative embodiment, the system comprises:

(a) microbeads having a sensing interface carrying two or more copies of a capturing moiety;

(b) a light microscope coupled to an optical image acquisition device;

(c) an image analysis device.

The invention also provides a kit for use in the method of the present invention comprising:

(a) at least one reagent comprising a recognition agent comprising at least two capturing moieties to which the analyte binds if present in the sample.

(b) a sample carrier.

The ***sample carrier*** according to the invention may include any device which can carry the sample subject of detection and on which the association between the capturing agent and the analyte may be performed. The carrier thus may be a microscope slide, or be a testing chamber, supplemented with a cover slide, to form a monolayer of the objects.

According to an alternative embodiment, the kit comprises:

(a) at least one reagent comprising microbeads having a sensing interface carrying two or more copies of a capturing agent;

(b) microscope slides.

DESCRIPTION OF THE FIGURES

In order to understand the invention and to see how it may be carried out in practice, a preferred embodiment will now be described, by way of non-limiting example only, with reference to the accompanying Figures, in which:

Figs. 1A-1C show light microscope images of plasma samples incubated with microbeads coated with multiple copies of an antibody directed against D-dimer. The plasma samples containing a very low level of D-dimer (Fig. 1A), an intermediate level of D-dimer (Fig. 1B) or a high level of D-dimer (Fig. 1C).

Fig. 2A-2C are bar representations of the size distribution of particulates formed as a result of binding of D-dimer to microbeads coated with antibodies directed against D-dimer. Microbeads coated with multiple copies of antibodies directed against D-dimer were incubated with samples containing low levels of D-dimer (Fig. 2A), intermediate levels of D-dimer (Fig. 2B) or high levels of D-dimer (Fig 2C).

Fig. 3 shows the average particulates size obtained as a result of titration of plasma samples containing different concentrations of D-Dimer.

Fig. 4 shows the average size of particulates obtained with heparin induced thrombocytopenia (HIT) samples by use of the Diamed kit with negative samples (n=4) as well as positive samples (n=10).

EXAMPLES

The following examples were performed using Latex-microbeads coated with an antibody directed against D-dimer (Biopool International, Umea Sweeden, Cat# 150709, Example 1) or polymer beads, coated with heparin/PF4 complexes (DiaMed-ID PaGIA [Particle Gel Immuno Assay], Cat # 050051, DiaMed AG, 1785 Cressier s/Morat, Switzerland, Example 2).

In general, plasma samples were incubated with microbeads coated with the specific capturing agents for a predetermined time period. After incubation, each

sample was placed on a light microscope slide and examined by a light microscope. Images of the resulting samples were captured by a video camera (CCD Camera) mounted on the microscope. The images thus obtained were analyzed by an image analysis software (Galai, Beit- Haemek, Israel), to determine the number of aggregates and the distribution of the particulate sizes formed as a results of aggregation. The distribution of the particulate size correlated with the concentration of the analyte in the tested specimen and with the number of complexes formed between capturing agents and analytes as a result of incubation.

Example 1

A D-dimer kit of Dade Behring Inc. was used in order to determine the presence of D-dimer in plasma samples and operated according to manufacturer's instructions. In this specific assay three plasma samples: (i) containing a very low level of D-dimer, (ii) containing an intermediate level of D-dimer or (iii) containing a high level of D-dimer were tested for the presence of D-dimer by the use of microbeads coated with antibodies directed against D-dimer. The microbeads were incubated with each sample for 1 minute after which the samples were transferred to microscope plates and analyzed as described above.

Figs. 1A-1C and 2A-2C show the results obtained. In particular, a microscope specimen taken from sample (i) after incubation with the microbeads, did not form substantial particulates as observed by the microscope (Fig. 1A). In addition, analysis of the image obtained from this specimen revealed that the average size of the particulates formed by complexing between D-dimer and the microbeads is $21.6\pm1.8\mu m^2$ (Fig. 2A).

A microscope specimen taken from sample (ii) containing intermediate levels of D-dimer produced aggregates visible by the microscope (Fig. 1B). In addition, analysis of the image obtained from this specimen revealed a shift in the distribution of the particulates size, with an average particulate size of $48.3\pm27.2\mu m^2$ (Fig. 2B).

Finally, a specimen taken from sample (iii) containing high levels of D-dimer produced aggregates also visible by the microscope (Fig. 1C) and analysis of the image obtained from this specimen revealed an additional shift in the distribution of the particulate size (as compared to Fig. 2B), with an average particulate size of 156±155μm^2 (Fig. 2C).

These results obtained by detection of aggregates size distribution correlate with the levels of D-dimer in the tested samples.

A titration curve of D-dimer concentrations in plasma samples was also determined. In particular, plasma samples containing different concentrations of D-dimer were incubated with anti-D-dimer antibody-coated beads for 1 minute after which specimens from the different samples were analyzed by the use of light microscope. Fig. 3 presents the titration curve obtained immediately after incubation period terminated and shows that there is a direct correlation between the D-dimer concentration in the samples and the average size of the aggregates formed as a result of complexing between D-dimer molecules present in the sample and the immunobeands with which the sample was incubated. These results suggest the use of the method of the invention not only for qualitative determination but also as an analytical tool for quantitative determinations.

Example 2

HIT syndrome results from an immune response to complex of heparin and platelet factor 4 (PF-4), which is located on the surface of platelet membrane, in some patients while treated by heparin. The result of this response is an immune mediated thrombocytopenia, or, in fewer cases, also thrombosis of the skin or other organs. In the following assay beads coated with heparin and PF-4 are used, which interact with a patient's plasma. In the case of a positive response, aggregates of beads are captured.

To this end, a HIT kit of Diamed (DiaMed, Cressier, Switzerland), was used

in this assay and operated according to manufacturer's instructions in order to determine positive and negative samples. In general, plasma samples were mixed with ID-PaGIA polymer particles, at a ratio of 5:1, and incubated at room temperature for 5 minutes. Specimens from each sample were obtained for further analysis as described above.

Fourteen plasma samples were tested, 4 of which were negative and 10 positive according to Diamed kit. The average size of the particulates obtained by the image analyzer is presented in Fig. 4, which shows that the average size of the particulates in the negative control group was $11.6\pm1.2\mu m^2$ while in the positive group $39.7\pm4.4\mu m^2$. Unpaired student-t test analysis of the data demonstrated a significant difference between the negative control group and the positive group of $p<0.002$.

CLAIMS

1. A method for detecting an analyte in a sample, the analyte comprising at least two recognition sites, the method comprising:

(a) providing a capturing agent comprising at least two capturing moieties;

(b) contacting the sample with said agent under conditions so as to allow the analyte, if present in the sample, to associate with the capturing agent via binding of a recognition site of said analyte to a capturing moiety of said agent, thereby forming agent-analyte particulates;

(c) obtaining an optical image of the sample by a light microscope; and

(d) determining from the image the absence or presence of particulates formed as a result of the binding between said analyte and said capturing agent, the presence of particulates in the sample indicating the presence of said analyte in the sample.

2. A method for detecting an analyte in a sample, the analyte comprising at least two recognition sites, the method comprising:

(a) providing a capturing agent comprising at least two capturing moieties;

(b) contacting the sample with said capturing agent under conditions so as to allow the analyte, if present in the sample, to associate with the capturing agent via binding of a recognition site of said analyte to a capturing moiety of said agent, thereby forming agent-analyte particulates;

(c) obtaining an optical image of the sample by a light microscope; and

(d) determining from the image size distribution of the particulates formed as a result of the binding between said analyte and said capturing agent, the size distribution of the particulates indicating level of analyte in the sample.

3. The method of Claim 1 or 2, wherein said agent comprises at least two antigenic epitopes and said analyte is an antibody comprising at least two recognition sites to which antigenic epitopes bind.

4. The method of Claim 1 or 2, wherein said agent is an antibody comprising at least two recognition sites and the analyte comprises at least two antigenic epitopes to which the antibody binds.

5. The method of Claim 1 or 2, wherein said analyte is a particle comprising on its surface two or more copies of a recognition site.

6. The method of Claim 5, wherein said particle is a cell or a microorganism presenting on their outer surface said recognition sites.

7. The method of Claim 6, wherein the recognition site is an antigen and said capturing agent is an antibody comprising at least two capturing moieties to which an antigenic epitope of said antigen binds.

8. A method for detecting an analyte in a sample, the analyte comprising at least two recognition sites, comprising:

(a) providing capturing agents comprising microbeads having a sensing interface carrying two or more copies of a capturing moiety;

(b) contacting the sample with the microbeads under conditions so as to allow the analyte, if present in the sample, to bind to a capturing moiety;

(c) obtaining an optical image of the sample by a light microscope and

(d) determining from the image the absence or presence of particulates formed as a result of the binding between said analyte and said capturing agent, the presence of said particulates indicating the presence of said analyte in the sample.

9. A method for detecting an analyte in a sample, comprising:

(a) providing capturing agents comprising microbeads having a sensing interface carrying two or more copies of a capturing moiety;

(b) contacting the sample with the microbeads under conditions so as to allow the analyte, if present in the sample, to bind to the capturing moiety;

(c) obtaining an optical image of the sample by a light microscope and

(d) determining from the image size distribution of particulates formed as

a result of the binding between said analyte and said capturing moiety, the size distribution of the particulates indicating the level of analyte in the sample.

10. The method of Claim 8 or 9, wherein said sensing interface carries two or more copies of a single capturing moiety.

11. The method of Claim 8 or 9, wherein said sensing interface carries two or more copies of different capturing moieties.

12. The method of Claim 8 or 9, wherein said analyte is an antibody comprising at least two recognition sites and said capturing agent comprises an antigenic epitope to which said antibody binds.

13. The method of Claim 8 or 9, wherein said analyte is an antigen comprising at least two antigenic epitopes and said capturing agent comprises antibodies or fragments of antibodies which bind to said antigen.

14. A system for detecting an analyte in a sample, comprising:

(a) capturing agents comprising two or more capturing moieties for binding to said analyte;

(b) a light microscope coupled to an optical image acquisition device;

(c) an image analysis device.

15. A system for detecting an analyte in a sample, comprising:

(a) microbeads having a sensing interface carrying two or more copies of a capturing moiety;

(b) a light microscope coupled to an optical image acquisition device;

(c) an image analysis device.

16. A kit for detection of an analyte in a sample according to the method of Claims 1 to 13, comprising:

(a) at least one reagent comprising a capturing agent comprising at least two capturing moieties to which the analyte binds if present in the sample.

(b) a sample carrier.

17. A kit for detection of an analyte in a sample according to the method of Claims 1 to 13, comprising:

(a) at least one reagent comprising microbeads having a sensing interface carrying two or more copies of a capturing agent;

(b) a sample carrier.

18. The kit of Claim 17 or 18, wherein said sample carrier is a microscope slide or a testing chamber supplemented with a cover slide.

ABSTRACT

The present invention concerns a rapid and easy method of diagnosing *in vitro* an analyte in a sample obtained from a subject, without the need of any sophisticated equipment.

The method of the invention comprises in general detection of an analyte in a sample, the analyte comprising at least two recognition sites, and the method comprises providing a capturing agent comprising at least two capturing moieties capable of binding to a recognition site of said analyte; contacting the sample with said capturing agent under conditions so as to allow the analyte, if present in the sample, to associate with the agent via binding of a recognition site of said analyte to a capturing moiety of said agent, thereby forming agent-analyte particulate; obtaining an optical image of the sample by a light microscope and qualitative or quantitative determining from the image the absence or presence of particulates formed as a result of the binding between said analyte and said capturing agent.

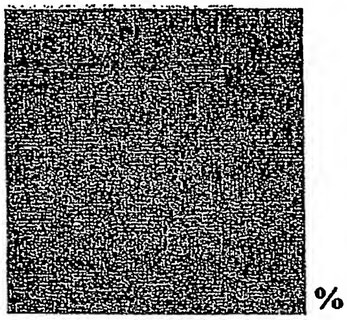

Figure 1A

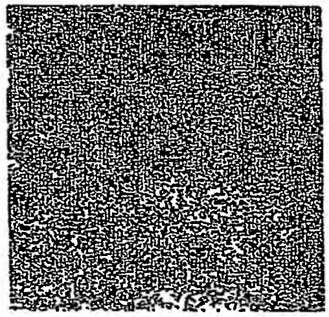

Figure 1B

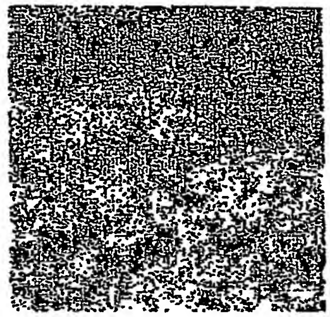

Figure 1C

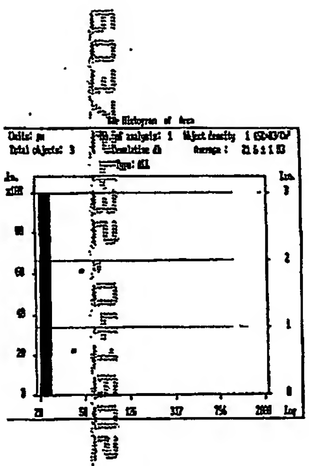

Figure 2A

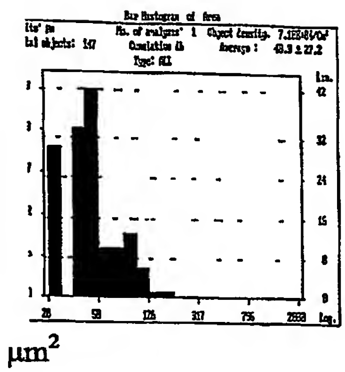

Figure 2B

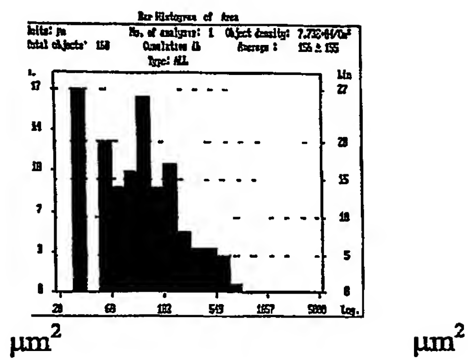

Figure 2C

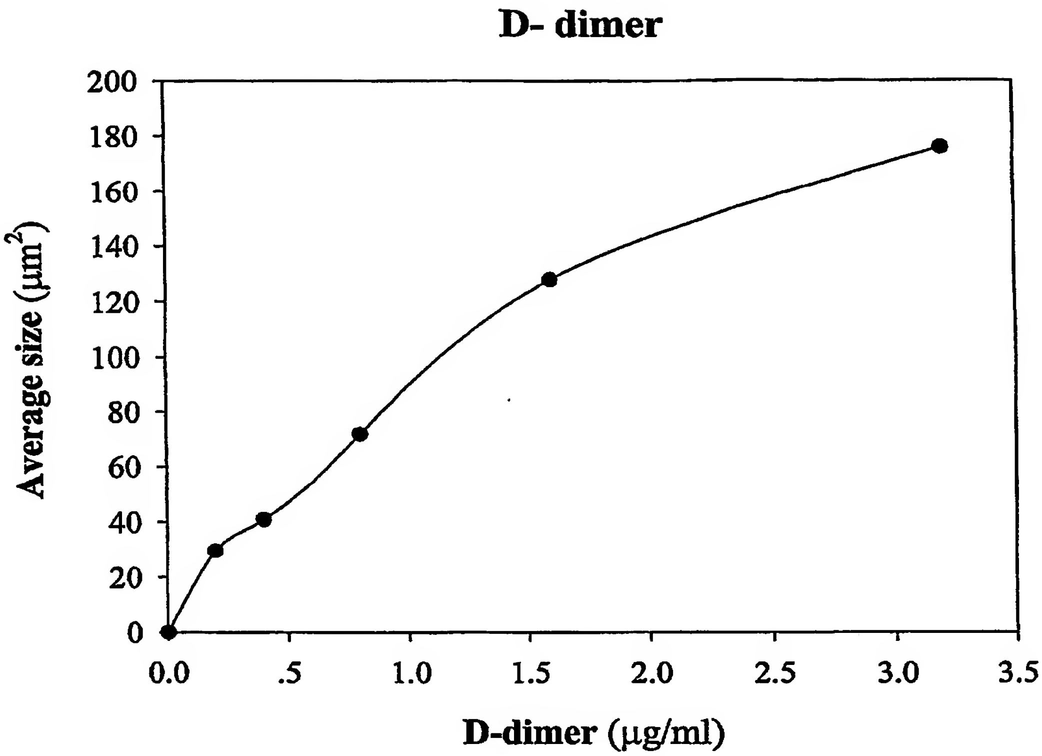

Figure 3

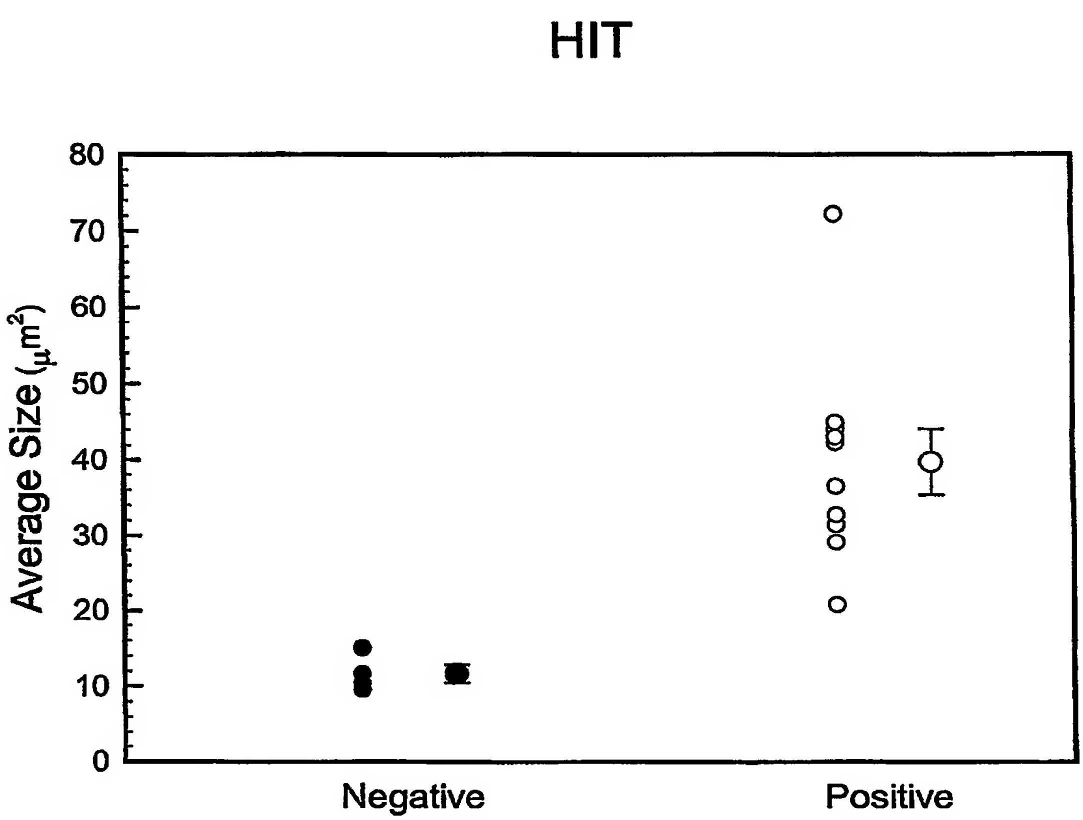

Figure 4